INTERACTION OF NMDA RECEPTOR WITH PROTEIN TYROSINE PHOSPHATASE

BACKGROUND OF THE INVENTION

[01] In the majority of mammalian excitatory synapses, glutamate (Glu) mediates rapid chemical neurotransmission by binding to three distinct types of glutamate receptors on the surfaces of brain neurons. Although cellular responses mediated by glutamate receptors are normally triggered by exactly the same excitatory amino acid (EAA) neurotransmitters in the brain (e.g., glutamate or aspartate), the different subtypes of glutamate receptors have different patterns of distribution in the brain, and mediate different cellular signal transduction events. One major class of glutamate receptors is referred to as N-methyl-D-aspartate receptors (NMDA-Rs), since they bind preferentially to N-methyl-D-aspartate (NMDA). NMDA is a chemical analog of aspartic acid; it normally does not occur in nature, and NMDA is not present in the brain. When molecules of NMDA contact neurons having NMDA-Rs, they strongly activate the NMDA-R (i.e., they act as a powerful receptor agonist), causing the same type of neuronal excitation that glutamate does. It has been known that excessive activation of NMDA-R plays a major role in a number of important central nervous system (CNS) disorders, while hypoactivity of NMDA-R has been implicated in several psychiatric diseases.

[02] NMDA-Rs contain an NR1 subunit and at least one of four different NR2 and NR3 subunits (designated as NR2A, NR2B, NR2C, and NR2D, NR3A and NR3B). NMDA-Rs are "ionotropic" receptors since they flux ions, such as Ca2+. These ion channels allow ions to flow into a neuron upon depolarization of the postsynaptic membrane. , when the receptor is activated by glutamate, aspartate, or an agonist drug.

[03] Protein tyrosine phosphorylation plays an important role in regulating diverse cellular processes. The regulation of protein tyrosine phosphorylation is mediated by the reciprocal actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). NMDA-Rs are regulated by protein tyrosine kinases and phosphatases. Phosphorylation of NMDA-R by protein tyrosine kinases results in enhanced NMDA-R responsiveness in neurons (Wang et al., Nature 369:233-235, 1994). NR2B and NR2A have been shown to be the main sites of phosphorylation by protein tyrosine kinases. Protein tyrosine phosphatases, on the other hand, exert opposing effects on the responsiveness of NMDA-R in the neurons (Wang et al, Proc. Natl. Acad. Sci. U.S.A. U.S.A. 93:1721-1725, 1996). It is believed that members of the Src family of protein tyrosine kinases mediate the NMDA-R

tyrosine phosphorylation. On the other hand, the identity of the enzyme responsible for the counter dephosphorylation of NMDA-R has been elusive.

SUMMARY OF THE INVENTION

[04] Methods are provided for identifying a modulator of N-methyl-D-aspartate receptor (NMDA-R) signaling by detecting the ability of an agent to modulate the phosphatase activity of a protein tyrosine phosphatase (PTP), *e.g.* on a NMDA-R substrate, on a kinase in a signaling pathway associated with NMDA-R, *etc.*, or to modulate the binding of the PTP to NMDA-R. In one embodiment, the modulator is identified by detecting its ability to modulate the phosphatase activity of the PTP. In another embodiment, the modulator is identified by detecting its ability to modulate the binding of the PTP and the NMDA-R. In another embodiment, methods are provided for identifying a nucleic acid molecule encoding polypeptides that modulate NMDA-R signaling.

[05] Methods are provided for treating a disease associated with abnormal NMDA-R-signaling by administering a modulator of a PTP activity, which directly or indirectly modulates the tyrosine phosphorylation level of the NMDA-R. The modulator may affect the ability of the PTP to dephosphorylate NMDA-R, to dephosphorylate kinases in a signaling pathway associated with NMDA-R, and/or the ability of the PTP to bind to NMDA-R. In certain embodiments, the modulator is a PTP agonist and the disease to be treated is mediated by excessive NMDA-R signaling. In other embodiments, the modulator is a PTP antagonist and the disease to be treated is mediated by NMDA-R hypofunction.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[06] The present invention relates to the discovery of a binding interaction between the NR2A or NR2B subunits of the NMDA-R and protein tyrosine phosphatase. In accordance with the discovery, the present invention provides methods for identifying agonists and antagonists of PTPs that modulate NMDA-R signaling, and for treating conditions mediated by abnormal NMDA-R signaling. The following description provides guidance for making and using the compositions of the invention, and for carrying out the methods of the invention.

DEFINITIONS

[07] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention pertains. The following references provide one of skill with a general definition of

many of the terms used in this invention: Singleton *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY (2d ed. 1994); THE CAMBRIDGE DICTIONARY OF SCIENCE AND TECHNOLOGY (Walker ed., 1988); and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY (1991). Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. The following definitions are provided to assist the reader in the practice of the invention.

[08] As used herein, the term "acute insult to the central nervous system" includes short-term events that pose a substantial threat of neuronal damage mediated by glutamate excitotoxicity. These include ischemic events (which involve inadequate blood flow, such as a stroke or cardiac arrest), hypoxic events (involving inadequate oxygen supply, such as drowning, suffocation, or carbon monoxide poisoning), trauma to the brain or spinal cord (in the form of mechanical or similar injury), certain types of food poisoning which involve an excitotoxic poison such as domoic acid, and seizure-mediated neuronal degeneration, which includes certain types of severe epileptic seizures. It can also include trauma that occurs to another part of the body, if that trauma leads to sufficient blood loss to jeopardize blood flow to the brain (for example, as might occur following a shooting, stabbing, or automobile accident).

[09] The term "agent" includes any substance, molecule, element, compound, entity, or a combination thereof. It includes, but is not limited to, e.g., protein, oligopeptide, small organic molecule, polysaccharide, polynucleotide, and the like. It can be a natural product, a synthetic compound, or a chemical compound, or a combination of two or more substances. Unless otherwise specified, the terms “agent”, “substance”, and “compound” can be used interchangeably.

[10] As used herein, an "agonist" is a molecule which, when interacting with (e.g., binding to) a target protein (e.g., PTPL1, NMDA-R), increases or prolongs the amount or duration of the effect of the biological activity of the target protein. By contrast, the term "antagonist," as used herein, refers to a molecule which, when interacting with (e.g., binding to) a target protein, decreases the amount or the duration of the effect of the biological activity of the target protein (e.g., PTPL1 or NMDA-R). Agonists and antagonists may include proteins, nucleic acids, carbohydrates, antibodies, or any other molecules that decrease the effect of a protein. Unless otherwise specified, the term “agonist” can be used interchangeably with “activator”, and the term “antagonist” can be used interchangeably with “inhibitor”.

[11] The term "analog" is used herein to refer to a molecule that structurally resembles a molecule of interest but which has been modified in a targeted and controlled manner, by replacing a specific substituent of the reference molecule with an alternate substituent. Compared to the starting molecule, an analog may exhibit the same, similar, or improved utility. Synthesis and screening of analogs, to identify variants of known compounds having improved traits (such as higher potency at a specific receptor type, or higher selectivity at a targeted receptor type and lower activity levels at other receptor types) is an approach that is well known in pharmaceutical chemistry.

[12] The term "biological preparation" refers to biological samples taken *in vivo* and *in vitro* (either with or without subsequent manipulation), as well as those prepared synthetically. Representative examples of biological preparations include cells, tissues, solutions and bodily fluids, a lysate of natural or recombinant cells.

[13] As used herein, the term "functional derivative" of a native protein or a polypeptide is used to define biologically active amino acid sequence variants that possess the biological activities (either functional or structural) that are substantially similar to those of the reference protein or polypeptide. Thus, a functional derivative of a PTP may retain, among other activities, the ability to bind to, and dephosphorylate NMDA-R. Similarly, a functional derivative of NMDA-R may be capable of binding to a PTP, and of being dephosphorylated by a PTP.

[14] NMDA receptors are a subclass of excitatory, ionotropic L-glutamate neurotransmitter receptors. They are heteromeric, integral membrane proteins being formed by the assembly of the obligatory NR1 subunit together with modulatory NR2 subunits. The NRI subunit is the glycine binding subunit and exists as 8 splice variants of a single gene. The glutamate binding subunit is the NR2 subunit, which is generated as the product of four distinct genes, and provides most of the structural basis for heterogeneity in NMDA receptors. In the hippocampus and cerebral cortex, the active subunit NMDAR1 is associated with 1 of 2 regulatory epsilon subunits: NMDAR2A or NMDAR2B and NR3. Unless otherwise specified, the term "NMDA-R" or "NMDA receptor" as used herein refers to an NMDA receptor molecule that has an NR1 subunit and at least one NR2A or NR2B subunit.

[15] An exemplary NR1 subunit is the human NMDAR1 polypeptide. The sequence of the polypeptide and corresponding nucleic acid may be obtained at Genbank, accession number L05666, and is published in Planells-Cases *et al.* (1993) P.N.A.S. **90**(11):5057-5061. An exemplary NR2 subunit is the human NMDAR2A polypeptide. The sequence of the polypeptide and corresponding nucleic acid may be obtained at Genbank, accession number

U09002, and is published in Foldes *et al.* (1994) Biochim. Biophys. Acta 1223 (1):155-159. Another NR2 subunit is the human NMDAR2B polypeptide. The sequence of the polypeptide and corresponding nucleic acid may be obtained at Genbank, accession number U11287, and is published in Adams *et al.* (1995) Biochim. Biophys. Acta 1260 (1):105-108.

[16] Protein tyrosine phosphatases of the invention are characterized by an association with NMDA-R *in vivo*, particular in neural tissue, more particularly in brain tissue. A fundamental process for regulating the function of NMDA receptors and other ion channels in neurons is tyrosine phosphorylation. A phosphatase enzyme may act on NMDA-R directly, to dephosphorylate one or more of the NMDA-R subunits. Alternatively a phosphatase enzyme may act on NMDA-R indirectly, by dephosphorylating a protein tyrosine kinase (PTK) in a signaling pathway. For example, a phosphatase that acts to decrease the activity of a PTK that phosphorylates NMDA-R, will indirectly result in decreased phosphorylation of NMDA-R.

[17] PTPL1 refers to a protein tyrosine phosphatase, also known as PTPN13. An exemplary PTPL1 molecule is the human polypeptide. The sequence of the polypeptide and corresponding nucleic acid may be obtained at Genbank, accession number X80289, and is published by Saras *et al.* (1994) J. Biol. Chem. **269** (39):24082-24089.

[18] PTP MEG refers to a protein tyrosine phosphatase, also known as PTPN3. An exemplary PTP MEG molecule is the human polypeptide. The sequence of the polypeptide and corresponding nucleic acid may be obtained at Genbank, accession number NM_002830.

[19]

[20] PTKs have been found to potentiate the function of recombinant NMDA receptors. The family of Src kinases comprises a total of nine members, five of which Src, Fyn, Lyn, Lck, and Yes are known to be expressed in the CNS. All members of the Src family contain highly homologous regions the C-terminal, catalytic, Src homology 2, and Src homology 3 domains. The kinase activity of Src protein is normally inactivated by phosphorylation of the tyrosine residue at position 527, which is six residues from the C-terminus. Hydrolysis of phosphotyrosine 527 by a phosphatase enzyme normally activates c-Src.

[21] As used herein, the term "NMDA-R signaling" refers to signal-transducing activities in the central nervous system that are involved in the various cellular processes such as neurodevelopment, neuroplasticity, and excitotoxicity. NMDA-R signaling affects a variety of processes including, but not limited to, neuron migration, neuron survival, synaptic maturation, learning and memory, and neurodegeneration.

[22] The term "NMDA-R hypofunction" is used herein to refer to abnormally low levels of signaling activity of NMDA-Rs on CNS neurons. For example, NMDA-R hypofunction may be caused by abnormally low phosphotyrosine level of NMDA-R. NMDA-R hypofunction can occur as a drug-induced phenomenon. It can also occur as an endogenous disease process.

[23] The term "modulation" as used herein refers to both upregulation, (i.e., activation or stimulation), for example by agonizing; and downregulation (i.e. inhibition or suppression), for example by antagonizing, of a bioactivity (e.g., direct or indiriect NMDA-R tyrosine phosphorylation, PTPL1 tyrosine phosphatase activity, PTPL1 binding to NMDA-R). As used herein, the term "modulator of NMDA-R signaling" refers to an agent that is able to alter an NMDA-R activity that is involved in the NMDA-R signaling pathways. Modulators include, but are not limited to, both "activators" and "inhibitors" of NMDA-R tyrosine phosphorylation. An "activator" is a substance that directly or indirectly enhances the tyrosine phosphorylation level of NMDA-R, and thereby causes the NMDA receptor to become more active. The mode of action of the activator may be direct, e.g., through binding the receptor, or indirect, e.g., through binding another molecule which otherwise interacts with NMDA-R (e.g., PTPL1, Src, Fyn, *etc*). Conversely, an "inhibitor" directly or indirectly decreases the tyrosine phosphorylation of NMDA-R, and thereby causes NMDA receptor to become less active. The reduction may be complete or partial. As used herein, modulators of NMDA-R signaling encompass PTPL1 antagonists and agonists.

[24] As used herein, the term "PTP modulator" includes both "activators" and "inhibitors" of PTP phosphatase activity. An "activator" of PTP is a substance that causes a PTP to become more active, and thereby directly or indirectly decreases the phosphotyrosine level of NMDA-R. The mode of action of the activator may be through binding the PTP; through binding another molecule which otherwise interacts with the PTP; *etc.* Conversely, an "inhibitor" of a PTP is a substance that causes the PTP to become less active, and thereby directly or indirectly increases phosphotyrosine level of NMDA-R. The reduction may be complete or partial, and due to a direct or an indirect effect.

[25] As used herein, the term "polypeptide containing the PDZ2 domain of a PTP" includes the PTP, and other polypeptides that contain the PDZ2 domain, or their derivatives, analogs, variants, or fusion proteins that can bind to NR2A and/or NR2B. The term "polypeptide containing a PTP -binding site of NMDA-R" include an NMDA-R that has at least an NR2A or NR2B subunit, NR2A, NR2B, and other polypeptides that contain the PTP -

binding site of NR2A or NR2B, or their derivatives, analogs, variants, or fusion proteins that can bind to PTP.

[26] PDZ domains are modular protein interaction domains that bind in a sequence-specific fashion to short C-terminal peptides or internal peptides that fold in a ß-finger. PDZ domains typically comprise GLGF repeats. PDZ domains are relatively small (>90 residues), fold into a compact globular fold and have N- and C-termini that are close to one another in the folded structure. The PDZ fold consists of six ß-strands and two α-helices. Peptide ligands bind in an extended groove between strand ßB and helix αB by a mechanism referred to as ß-strand addition. Specifically, the peptide serves as an extra ß-strand that is added onto the edge of a pre-existing ß-sheet within the PDZ domain. The peptide ligand backbone participates in the extensive hydrogen-bonding pattern normally observed between main-chain carbonyl and amide groups in a ß-sheet structure. The structure of the PDZ domain does not change upon ligand binding.

[27] The architecture of the PDZ domain is designed for binding to a free carboxylate group at the end of the peptide. The carboxylate-binding loop lies between the ßA and ßB strands, extending from a highly conserved arginine or lysine residue to the signature Gly-Leu-Gly-Phe (GLGF) motif. Three main-chain amide protons of the GLGF motif form hydrogen bonds with the terminal carboxylate of the peptide. Since a free carboxylate group occurs only at the very C terminus of the peptide main chain, the interactions between the carboxylate-binding loop and the carboxylate oxygens form the structural basis for PDZ recognition of C-terminal peptides. The carboxylate-binding loop (R/K-XXX-GLGF) is highly conserved among PDZ domains. The second and fourth residues of the GLGF motif are invariably hydrophobic. The second of the two glycines is absolutely conserved, but a serine, threonine, or proline replaces the first glycine in a minority of PDZs. Examples of PDZ domains are reviewed in Sheng and Sala (2001) Annu. Rev. Neurosci. **24**:1-29, and Ponting *et al.* (1997) Bioessays 19:469-479.

[28] As used herein, the term "PTP /NMDA-R-containing protein complex" refers to protein complexes, formed *in vitro* or *in vivo*, that contain PTP and NMDA-R. When only the binding of PTP and NMDA-R is of concern, a polypeptide containing the PDZ2 domain of PTP and a polypeptide containing PTP -binding site of NMDA-R can substitute for the PTP and NMDA-R respectively. However, when dephosphorylation of NMDA-R is in concern, only a functional derivative and an NMDA-R functional derivative as defined herein can respectively substitute for the PTP and NMDA-R in the complex. In addition, the complex may also comprise other components, e.g., a protein tyrosine kinase such as Fyn, Src, *etc.*

[29] The terms "substantially pure" or "isolated," when referring to proteins and polypeptides, *e.g.*, a fragment of a PTP, denote those polypeptides that are separated from proteins or other contaminants with which they are naturally associated. A protein or polypeptide is considered substantially pure when that protein makes up greater than about 50% of the total protein content of the composition containing that protein, and typically, greater than about 60% of the total protein content. More typically, a substantially pure or isolated protein or polypeptide will make up at least 75%, more preferably, at least 90%, of the total protein. Preferably, the protein will make up greater than about 90%, and more preferably, greater than about 95% of the total protein in the composition.

[30] A "variant" of a molecule such as a PTP or NMDA-R is meant to refer to a molecule substantially similar in structure and biological activity to either the entire molecule, or to a fragment thereof. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein if the composition or secondary, tertiary, or quaternary structure of one of the molecules is not identical to that found in the other, or if the sequence of amino acid residues is not identical.

[31] As used herein, "recombinant" has the usual meaning in the art, and refers to a polynucleotide synthesized or otherwise manipulated *in vitro* (e.g., "recombinant polynucleotide"), to methods of using recombinant polynucleotides to produce gene products in cells or other biological systems, or to a polypeptide ("recombinant protein") encoded by a recombinant polynucleotide.

[32] The term "operably linked" refers to functional linkage between a nucleic acid expression control sequence (such as a promoter, signal sequence, or array of transcription factor binding sites) and a second polynucleotide, wherein the expression control sequence affects transcription and/or translation of the second polynucleotide.

[33] The term "recombinant" when used with reference to a cell indicates that the cell replicates a heterologous nucleic acid, or expresses a peptide or protein encoded by a heterologous nucleic acid. Recombinant cells can contain genes that are not found within the native (non-recombinant) form of the cell. Recombinant cells can also contain genes found in the native form of the cell wherein the genes are modified and re-introduced into the cell by artificial means. The term also encompasses cells that contain a nucleic acid endogenous to the cell that has been modified without removing the nucleic acid from the cell; such modifications include those obtained by gene replacement, site-specific mutation, and related techniques.

[34] A "heterologous sequence" or a "heterologous nucleic acid," as used herein, is one that originates from a source foreign to the particular host cell, or, if from the same source, is modified from its original form. Thus, a heterologous gene in a prokaryotic host cell includes a gene that, although being endogenous to the particular host cell, has been modified. Modification of the heterologous sequence can occur, *e.g.*, by treating the DNA with a restriction enzyme to generate a DNA fragment that is capable of being operably linked to the promoter. Techniques such as site-directed mutagenesis are also useful for modifying a heterologous nucleic acid.

[35] A "recombinant expression cassette" or simply an "expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, that has control elements that are capable of affecting expression of a structural gene that is operably linked to the control elements in hosts compatible with such sequences. Expression cassettes include at least promoters and optionally, transcription termination signals. Typically, the recombinant expression cassette includes at least a nucleic acid to be transcribed (*e.g.*, a nucleic acid encoding a PTP) and a promoter. Additional factors necessary or helpful in effecting expression can also be used as described herein. For example, transcription termination signals, enhancers, and other nucleic acid sequences that influence gene expression, can also be included in an expression cassette.

[36] As used herein, "contacting" has its normal meaning and refers to combining two or more agents (e.g., two proteins, a polynucleotide and a cell, etc.). Contacting can occur *in vitro* (e.g., two or more agents [e.g., a test compound and a cell lysate] are combined in a test tube or other container) or *in situ* (e.g., two polypeptides can be contacted in a cell by coexpression in the cell, of recombinant polynucleotides encoding the two polypeptides), in a cell lysate "

[37] Various biochemical and molecular biology methods referred to herein are well known in the art, and are described in, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, N.Y. Second (1989) and Third (2000) Editions, and Current Protocols in Molecular Biology, (Ausubel, F.M. et al., eds.) John Wiley & Sons, Inc., New York (1987-1999).

SCREENING FOR MODULATORS OF NMDA-R SIGNALING

[38] The present invention provides methods for identifying modulators of NMDA-R signaling. The NMDA-R modulators are identified by detecting the ability of an agent to

modulate an activity of a protein tyrosine phosphatase (PTP), which is capable of directly or indirectly dephosphorylating an NMDA-R. The modulated activities of the PTP include, but are not limited to, its phosphatase activity or its binding to NMDA-R.

[39] In one aspect, NMDA-R modulators of the present invention are identified by monitoring their ability to modulate phosphatase activity. As will be detailed below, PTP, the NMDA-R/ PTP -containing protein complex, or cell lines that express a PTP or NMDA-R/ PTP -containing protein complex, are used to screen for PTP agonists and antagonists that modulate direct or indirect NMDA-R tyrosine dephosphorylation, e.g. in the presence of a protein tyrosine kinase in a signaling pathway with a PTP and NMDA-R. An agent that enhances the ability of A PTP to directly or indirectly dephosphorylate NMDA-R will result in a net decrease in the amount of phosphotyrosine, whereas an agent that inhibits the ability of A PTP to directly or indirectly dephosphorylate NMDA-R will result in a net increase in the amount of phosphotyrosine.

[40] In some embodiments, the ability of an agent to enhance or inhibit A PTP phosphatase activity is assayed in an *in vitro* system. In general, the *in vitro* assay format involves adding an agent to A PTP (or a functional derivative of A PTP) and a substrate of A PTP, *e.g.* Src, Fyn, *etc.*, and measuring the tyrosine phosphorylation level of the substrate. In one embodiment, as a control, tyrosine phosphorylation level of the substrate is also measured under the same conditions except that the test agent is not present. By comparing the tyrosine phosphorylation levels of the substrate, PTP antagonists or agonists can be identified. Specifically, a PTP antagonist is identified if the presence of the test agent results in an increased tyrosine phosphorylation level of the substrate. Conversely, a decreased tyrosine phosphorylation level in the substrate indicates that the test agent is a PTP agonist. The invention provides the use of such agents to modulate NMDA-R activity.

[41] PTP used in the assays is obtained from various sources. In some embodiments, PTP used in the assays is purified from cellular or tissue sources, e.g., by immunoprecipitation with specific antibodies. In other embodiments, as described below, PTP is purified by affinity chromatography utilizing specific interactions of PTP with known protein motifs, e.g., the interaction of the PDZ2 domain of a PTP with NR2A and/or NR2B. In still other embodiments, the PTP, either holoenzyme or enzymatically active parts of it, is produced recombinantly either in bacteria or in eukaryotic expression systems. The recombinantly produced variants of PTP scan contain short protein tags, such as immunotags (HA-tag, c-myc tag, FLAG-tag) , 6 x His-tag, GST tag, *etc.*, which could be used

to facilitate the purification of recombinantly produced PTP using immunoaffinity or metal-chelation-chromatography, respectively.

[42] Various substrates are used in the assays. Preferably, the substrate is Src, Fyn, NMDA-R, a functional derivative of NMDA-R, or the NR2A or NR2B subunit. In some embodiments, the substrates used are proteins purified from a tissue (such as immunoprecipitated NR2A or NR2B from rat brain). In other embodiments, the substrates are recombinantly expressed proteins. Examples of recombinant substrates include, but are not limited to, proteins expressed in *E. coli,* yeast, or mammalian expression systems. In still other embodiments, the substrates used are synthetic peptides that are tyrosine phosphorylated by specific kinase activity, e.g., Src or Fyn kinases.

[43] Methods and conditions for expression of recombinant proteins are well known in the art. See, e.g., Sambrook, *supra*, and Ausubel, *supra*. Typically, polynucleotides encoding the phosphatase and/or substrate used in the invention are expressed using expression vectors. Expression vectors typically include transcriptional and/or translational control signals (e.g., the promoter, ribosome-binding site, and ATG initiation codon). In addition, the efficiency of expression can be enhanced by the inclusion of enhancers appropriate to the cell system in use. For example, the SV40 enhancer or CMV enhancer can be used to increase expression in mammalian host cells. Typically, DNA encoding a polypeptide of the invention is inserted into DNA constructs capable of introduction into and expression in an in vitro host cell, such as a bacterial (e.g., *E. coli, Bacillus subtilus*), yeast (e.g., *Saccharomyces*), insect (e.g., *Spodoptera frugiperda*), or mammalian cell culture systems. Mammalian cell systems are preferred for many applications. Examples of mammalian cell culture systems useful for expression and production of the polypeptides of the present invention include human embryonic kidney line (293; Graham et al., 1977, J. Gen. Virol. 36:59); CHO (ATCC CCL 61 and CRL 9618); human cervical carcinoma cells (HeLa, ATCC CCL 2); and others known in the art. The use of mammalian tissue cell culture to express polypeptides is discussed generally in Winnacker, FROM GENES TO CLONES (VCH Publishers, N.Y., N.Y., 1987) and Ausubel, supra. In some embodiments, promoters from mammalian genes or from mammalian viruses are used, e.g., for expression in mammalian cell lines. Suitable promoters can be constitutive, cell type-specific, stage-specific, and/or modulatable or regulatable (e.g., by hormones such as glucocorticoids). Useful promoters include, but are not limited to, the metallothionein promoter, the constitutive adenovirus major late promoter, the dexamethasone-inducible MMTV promoter, the SV40 promoter, and promoter-enhancer combinations known in the art.

[44] The substrate may or may not be already in a tyrosine phosphorylated state (Lau & Huganir, J. Biol. Chem., 270: 20036-20041, 1995). In the case of a nonphosphorylated starting material, the substrate is typically phosphorylated, e.g., using an exogenous tyrosine kinase activity such as Src or Fyn.

[45] A variety of standard procedures well known to those of skill in the art are used to measure the tyrosine phosphorylation levels of the substrates. In some embodiments, a phosphotyrosine-recognizing antibody-based assay is used, e.g., radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), as well as fluorescently labeled antibodies whose binding can be assessed from levels of emitted fluorescence. See, e.g., U.S. Patent No. 5,883,110; Mendoza et al., Biotechniques. 27: 778-788, 1999. In other embodiments, instead of immunoassays, the substrates are directly labeled with a radioactive phosphate group using kinases that carry out selective tyrosine phosphorylation (Braunwaler et al., Anal. Biochem. 234:23-26, 1996). The rate of removal of radioactive label from the labeled substrate can be quantitated in liquid (e.g., by chromatographic separation) or in solid phase (in gel or in Western blots).

[46] Comparing a tyrosine phosphorylation level under two different conditions (e.g., in the presence and absence of a test agent) sometimes includes the step of recording the level of phosphorylation in a first sample or condition and comparing the recorded level with that of (or recorded for) a second portion or condition.

[47] In some embodiments of the invention, other than adding PTP to a substrate (e.g., NR2A or NR2B), the *in vitro* assays are performed with an NMDA-R/ PTP -containing protein complex. Such protein complexes contain NMDA-R and PTP, or their functional derivatives. In addition, the complexes may also contain PTK and other molecules. The NMDA-R/ PTP -containing protein complexes may be obtained from neuronal cells using methods well known in the art, e.g., immunoprecipitation as described in Grant *et al.* (WO 97/46877). Tyrosine phosphorylation levels of the substrates are assayed with standard SDS-PAGE and immunoblot analysis.

[48] In other embodiments, NMDA-R signaling modulators of the present invention are identified using in vivo assays. Such *in vivo* assay formats usually entail culturing cells co-expressing a PTP and its substrate (e.g., NR2A or NR2B; e.g., recombinant forms of a PTP and/or NMDA-R subunit substrate(s)), adding an agent to the cell culture, and measuring tyrosine phosphorylation level of the substrate in the cells. In one embodiment, as a control, tyrosine phosphorylation level of the substrate in cells not exposed to the test agent is also measured or determined.

[49] In one embodiment, the *in vivo* screening system is modified from the method described in U.S. Patent No. 5,958,719. Using this screening system, intact cells that express a PTP and a substrate of a PTP (e.g., Src, Fyn, NMDA-R, NR2A, or NR2B) are first treated (e.g., by NMDA) to stimulate the substrate phosphorylation. The cells are then incubated with a substance that can penetrate into the intact cells and selectively inhibit further phosphorylation (e.g., by a PTK) of the substrate, *e.g.* NMDA-R. The degree of phosphorylation of the substrate is then determined by, for example, disrupting the cells and measuring phosphotyrosine level of the substrate according to methods described above, *e.g.* with standard SDS-PAGE and immunoblot analysis. The activity of the PTP is determined from the measured degree of phosphorylation of the substrate. An additional measurement is carried out in the presence of an agent. By comparing the degrees of phosphorylation, agonists or antagonist of PTP that modulate NMDA-R tyrosine phosphorylation are identified.

[50] In another embodiment, the present invention provides a method for identifying a nucleic acid molecule encoding a gene product that is capable of modulating the tyrosine phosphorylation level of NMDA-R. In one embodiment, a test nucleic acid is introduced into host cells coexpressing a PTP and NMDA-R or their functional derivatives. Methods for introducing a recombinant or exogenous nucleic acid into a cell are well known and include, without limitation, transfection, electroporation, injection of naked nucleic acid, viral infection, liposome-mediated transport (see, e.g., Dzau et al., 1993, Trends in Biotechnology 11:205-210; Sambrook, *supra*, Ausubel, *supra*). The cells are cultured so that the gene product encoded by the nucleic acid molecule is expressed in the host cells and interacts with a PTP and NMDA-R or their functional derivatives, followed by measuring the phosphotyrosine level of the NMDA-R. The effect of the nucleic acid on NMDA-R-signaling is determined by comparing NMDA-R phosphotyrosine levels measured in the absence or presence of the nucleic acid molecule.

[51] It will be appreciated by one of skill in the art that modulation of binding of PTP and NMDA-R may also affect the level of tyrosine phosphorylation in NMDA-R by the PTP. Therefore, agents identified from screening using the *in vivo* and *in vitro assay* systems described above may also encompass agents that modulate NMDA-R tyrosine phosphorylation by modulating the binding of the PTP and NMDA-R. In some embodiments of the invention, NMDA-R modulators are identified by directly screening for agents that promote or suppress the binding of PTP and NMDA-R. Agents thus identified may be further

examined for their ability to modulate NMDA-R tyrosine phosphorylation, using methods described above or standard assays well known in the art.

[52] PTP In one embodiment, modulators of the interaction between a PTP and NR2A or NR2B are identified by detecting their abilities to either inhibit the PTP and NMDA-R from binding (physically contacting) each other or disrupts a binding of the PTP and NMDA-R that has already been formed. The inhibition or disruption can be either complete or partial. In another embodiment, the modulators are screened for their activities to either promote a PTP and NMDA-R binding to each other, or enhance the stability of a binding interaction between a PTP and NMDA-R that has already been formed. In either case, some of the in vitro and in vivo assay systems discussed above for identifying agents which modulate the NMDA-R tyrosine phosphorylation level may be directly applied or readily modified to monitor the effect of an agent on the binding of NMDA-R and a PTP. For example, a cell transfected to coexpress a PTP and NMDA-R or receptor subunit, in which the two proteins interact to form an NMDA-R/ PTP -containing complex, is incubated with an agent suspected of being able to inhibit this interaction, and the effect on the interaction measured. In some embodiments, a polypeptide containing a PDZ2 domain of PTP and a polypeptide containing PTP -binding site of NMDA-R can substitute for the intact PTP and NMDA-R proteins, respectively, in the NMDA-R/ PTP -containing protein complexes. Any of a number of means, such as coimmunoprecipitation, is used to measure the interaction and its disruption.

[53] Although the foregoing assays or methods are described with reference to PTPL1 and NMDA-R, the ordinarily skilled artisan will appreciate that functional derivatives or subunits of various PTPs and NMDA-R may also be used. For example, in various embodiments, NR2A or NR2B is used to substitute for an intact NMDA-R in assays for screening agents that modulate binding of a PTP and NMDA-R. In a related embodiment, an NMDA-R, Src, Fyn, functional derivative is used for screening agents that modulate phosphatase activity. In another embodiment, a polypeptide containing the PDZ2 domain of a PTP is used for screening agents that modulate the binding of the PTP and NMDA-R.

[54] Further, in various embodiments, functional derivatives of PTP that have amino acid deletions and/or insertions and/or substitutions (e.g., conservative substitutions) while maintaining their catalytic activity and/or binding capacity are used for the screening of agents. A functional derivative is prepared from a naturally occurring or recombinantly expressed PTP and NMDA-R by proteolytic cleavage followed by conventional purification procedures known to those skilled in the art. Alternatively, the functional derivative is

produced by recombinant DNA technology by expressing only fragments of a PTP or NMDA-R in suitable cells. In one embodiment, the partial receptor or phosphatase polypeptides are expressed as fusion polypeptides. It is well within the skill of the ordinary practitioner to prepare mutants of naturally occurring NMDA/ PTP proteins that retain the desired properties, and to screen the mutants for binding and/or enzymatic activity. NR2A and NR2B derivatives that can be dephosphorylated typically comprise the cytoplasmic domain of the polypeptides, e.g., the C-terminal 900 amino acids or a fragment thereof.

[55] In some embodiments, cells expressing a PTP and NMDA-R may be used as a source of the PTP and/or NMDA-R, crude or purified, or in a membrane preparation, for testing in these assays. Alternatively, whole live or fixed cells may be used directly in those assays. Methods for preparing fixed cells or membrane preparations are well known in the art, see, e.g., U. S. Patent No. 4,996,194. The cells may be genetically engineered to coexpress a PTP and NMDA-R. The cells may also be used as host cells for the expression of other recombinant molecules with the purpose of bringing these molecules into contact with a PTP and/or NMDA-R within the cell.

THERAPEUTIC APPLICATIONS AND PHARMACEUTICAL COMPOSITIONS

[56] It is well known in the art that NMDA-R agonists and antagonists can be used to treat symptoms caused by abnormal NMDA-R signaling, *e.g.* acute insult of the central nervous system (CNS). Methods of treatment using pharmaceutical composition comprising NMDA agonists and/or NMDA antagonists have been described, e.g., in U.S. Patent No. 5,902,815. As discussed in detail below, the present invention provides pharmaceutical compositions containing PTP antagonists and/or agonists that modulate NMDA-R tyrosine phosphorylation. Such agonists and antagonists include, but are not limited to, agents that interfere with PTP gene expression, agents that modulate the ability of a PTP to bind to NMDA-R or to dephosphorylate NMDA-R. In one embodiment, a PTP antisense oligonucleotide is used as a PTP antagonist in the pharmaceutical compositions of the present invention. In addition, PTP inhibitors that inhibit dephosphorylation of NMDA-R are useful as NMDA-R signaling modulators (e.g., orthovanadate, Li et al., Biochim. Biophys. Acta. 1405:110-20, 1998).

[57] Abnormal NMDA-R activity elicited by endogenous glutamate is implicated in a number of important CNS disorders. In one aspect, the present invention provides modulators of PTP that, by modulating phosphotyrosine level of NMDA-R, can treat or alleviate symptoms mediated by abnormal NMDA-R signaling. Indications of interest include

mild cognitive impairment (MCI), which can progress to Alzheimer's disease (AD). Treatment with acetylcholinesterase inhibitors can provide for modest memory improvement. Cognitive enhancers may also find use for memory loss associated with aging, and in the general public.

[58] One important use for NMDA antagonist drugs involves the ability to prevent or reduce excitotoxic damage to neurons. In some embodiments, the PTP agonists of the present invention, which promote the dephosphorylation of NMDA-R, are used to alleviate the toxic effects of excessive NMDA-R signaling. In certain other embodiments, PTP antagonists of the present invention, which function as NMDA-R agonists, are used therapeutically to treat conditions caused by NMDA-R hypo-function, i.e., abnormally low levels of NMDA-R signaling in CNS neurons. NMDA-R hypofunction can occur as an endogenous disease process. It can also occur as a drug-induced phenomenon, following administration of an NMDA antagonist drug. In some related embodiments, the present invention provides pharmaceutical compositions containing PTP antagonists that are used in conjunction with NMDA antagonists, e.g., to prevent the toxic side effects of the NMDA antagonists.

[59] Excessive glutamatergic signaling has been causatively linked to the excitotoxic cell death during an acute insult to the central nervous system such as ischemic stroke (Choi et al., Annu Rev Neurosci. 13: 171-182, 1990; Muir & Lees, Stroke 26: 503-513, 1995). Excessive glutamatergic signaling via NMDA receptors has been implicated in the profound consequences and impaired recovery after the head trauma or brain injury (Tecoma et al., Neuron 2:1541-1545, 1989; McIntosh et al., J. Neurochem. 55:1170-1179, 1990). NMDA receptor-mediated glutamatergic hyperactivity has also been linked to the process of slow degeneration of neurons in Parkinson's disease (Loopuijt & Schmidt, Amino Acids, 14: 17-23, 1998) and Huntington's disease (Chen et al., J. Neurochem. 72:1890-1898, 1999). Further, elevated NMDA-R signaling in different forms of epilepsy have been reported (Reid & Stewart, Seizure 6: 351-359, 1997).

[60] Accordingly, PTP agonists of the present invention are used for the treatment of these diseases or disorders by stimulating the NMDA receptor-associated phosphatase activity (such as that of PTPL1) or by promoting the binding of a PTP to the NMDA receptor complex.

[61] The PTP agonists (NMDA-R antagonists) of the present invention can also be used to treat diseases where a mechanism of slow excitotoxicity has been implicated (Bittigau & Ikonomidou, J. Child. Neurol. 12: 471-485, 1997). These diseases include, but are not

limited to, spinocerebellar degeneration (e.g., spinocerebellar ataxia), motor neuron diseases (e.g., amyotrophic lateral sclerosis (ALS)), mitochondrial encephalomyopathies. The PTPL1 agonists of the present invention can also be used to alleviate neuropathic pain, or to treat chronic pain without causing tolerance or addiction (see, e.g., Davar et al., Brain Res. 553: 327-330, 1991).

[62] NMDA-R hypofunction have been causatively linked to schizophrenic symptoms (Tamminga, Crit. Rev. Neurobiol. 12: 21-36, 1998; Carlsson et al., Br. J. Psychiatry Suppl.: 2-6, 1999; Corbett et al., Psychopharmacology (Berl). 120: 67-74, 1995; Mohn et al., Cell 98: 427-436, 1999) and various forms of cognitive deficiency, such as dementias (e.g., senile and HIV-dementia) and Alzheimer's disease (Lipton, Annu. Rev. Pharmacol. Toxicol. 38:159-177, 1998; Ingram et al., Ann. N. Y. Acad. Sci. 786: 348-361, 1996; Müller et al., Pharmacopsychiatry. 28: 113-124, 1995). In addition, NMDA-R hypofunction is also linked to psychosis and drug addiction (Javitt & Zukin, Am J Psychiatry. 148: 1301-8, 1991). Further, NMDA-R hypofunction is also associated with ethanol sensitivity (Wirkner et al., Neurochem. Int. 35: 153-162, 1999; Yagi, Biochem. Pharmacol. 57: 845-850, 1999).

[63] NMDA-R hypofuction has also been linked to depression .....

[64] Using a PTP antagonist (NMDA-R agonists) described herein, the present invention provides methods for the treatment of Schizophrenia, psychosis, cognitive deficiencies, drug addiction, and ethanol sensitivity by antagonizing the activity of the NMDA-R-associated PTPs, and that of PTPL1 in particular, or by inhibiting the interaction between the PTP and the NR2A or NR2B subunit.

[65] The PTP agonists and antagonists of the present invention are directly administered under sterile conditions to the host to be treated. However, while it is possible for the active ingredient to be administered alone, it is often preferable to present it as a pharmaceutical formulation. Formulations typically comprise at least one active ingredient together with one or more acceptable carriers thereof. Each carrier should be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the patient. For example, the bioactive agent may be complexed with carrier proteins such as ovalbumin or serum albumin prior to their administration in order to enhance stability or pharmacological properties such as half-life. Furthermore, therapeutic formulations of this invention are combined with or used in association with other therapeutic agents.

[66] The therapeutic formulations are delivered by any effective means that could be used for treatment. Depending on the specific NMDA-R antagonist and/or NMDA-R agonist being

used, the suitable means include but are not limited to oral, rectal, nasal, pulmonary administration, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) infusion into the bloodstream.

[67] Therapeutic formulations are prepared by any methods well known in the art of pharmacy. See, *e.g.*, Gilman et al (eds.) (1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics (8th ed.) Pergamon Press; and (1990) Remington's Pharmaceutical Sciences (17th ed.) Mack Publishing Co., Easton, P.a.; Avis et al (eds.) (1993) Pharmaceutical Dosage Forms: Parenteral Medications Dekker, N.Y.; Lieberman et al. (eds.) (1990) Pharmaceutical Dosage Forms: Tablets Dekker, N.Y.; and Lieberman et al (eds.) (1990) Pharmaceutical Dosage Forms: Disperse Systems Dekker, N.Y. The therapeutic formulations can conveniently be presented in unit dosage form and administered in a suitable therapeutic dose. The preferred dosage and mode of administration of a PTPL1 agonist and/or antagonist will vary for different patients, depending upon factors that will need to be individually reviewed by the treating physician. As a general rule, the quantity of a PTPL1 agonist and/or antagonist administered is the smallest dosage which effectively and reliably prevents or minimizes the conditions of the patients.

[68] A suitable therapeutic dose is determined by any of the well known methods such as clinical studies on mammalian species to determine maximum tolerable dose and on normal human subjects to determine safe dosage. In human patients, since direct examination of brain tissue is not feasible, the appearance of hallucinations or other psychotomimetic symptoms, such as severe disorientation or incoherence, should be regarded as signals indicating that potentially neurotoxic damage is being generated in the CNS by an NMDA-R antagonist. Additionally, various types of imaging techniques (such as positron emission tomography and magnetic resonance spectroscopy, which use labeled substrates to identify areas of maximal activity in the brain) may also be useful for determining preferred dosages of NMDA-R agonists for use as described herein, with or without NMDA-R antagonists.

[69] It is also desirable to test rodents or primates for cellular manifestations in the brain, such as vacuole formation, mitochondrial damage, heat shock protein expression, or other pathomorphological changes in neurons of the cingulate and retrosplenial cerebral cortices. These cellular changes can also be correlated with abnormal behavior in lab animals.

[70] Except under certain circumstances when higher dosages may be required, the preferred dosage of a PTP agonist and/or antagonist will usually lie within the range of from about 0.001 to about 1000 mg, more usually from about 0.01 to about 500 mg per day. It should be understood that the amount of any such agent actually administered will be

determined by a physician, in the light of the relevant circumstances that apply to an individual patient (including the condition or conditions to be treated, the choice of composition to be administered, including the particular PTP agonist or the particular PTP antagonist, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the chosen route of administration). Therefore, the above dosage ranges are intended to provide general guidance and support for the teachings herein, but are not intended to limit the scope of the invention.

[71] It is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, animal species or genera, constructs, and reagents described, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which scope will be determined by the language in the claims.

[72] It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a mouse" includes a plurality of such mice and reference to "the cytokine" includes reference to one or more cytokines and equivalents thereof known to those skilled in the art, and so forth.

[73] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

[74] All publications mentioned herein are incorporated herein by reference for all relevant purposes, e.g., the purpose of describing and disclosing, for example, the cell lines, constructs, and methodologies that are described in the publications which might be used in connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

[75] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the subject invention, and are not intended to limit the scope of what is regarded as the invention. Efforts have

been made to ensure accuracy with respect to the numbers used (*e.g.* amounts, temperature, concentrations, *etc.*) but some experimental errors and deviations should be allowed for. Unless otherwise indicated, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees centigrade; and pressure is at or near atmospheric.

EXPERIMENTAL

Example 1

Identification of Interaction between NMDA-R and PTPL1

Yeast two-hybrid screen.

[76] *NR2B interaction.* A yeast two-hybrid screen was carried out as follows. A commercially available human fetal brain cDNA library in the pACT2 vector pretransformed to the Y187 yeast strain (Clontech) was used. The cDNA corresponding to the 600 C-terminal amino acid residues of the NR2B subunit was fused with GAL4 BD by cloning it into the pAS2-1 vector (Clontech). The resulting GAL4BD-NR2B plasmid (bait) was transformed to Y190 strain (Clontech) to screen for the NR2B C-terminus interacting proteins in the human fetal brain cDNA library. Approximately 50 x 10^6 Y187 cells were mated in rich (YPD) medium for 20 hours with at least a ten-fold excess of Y190 cells carrying the bait vector. For selection of interactors, the yeast cells were plated for selection after mating on the solid yeast medium depleted of histidine and adenine. The AD plasmids from only those colonies which survived the double growth-selection and yielded strong colorimetric reaction in the β-galactosidase assay were further analyzed by DNA sequencing. Two yeast colonies contained identical cDNA clones which, in frame with the GAL4 AD, coded for the PDZ2 domain of protein tyrosine phosphatase PTPL1 together with some flanking sequence (127 amino acids N-terminally and 36 amino acids C-terminally). These results demonstrated that the PDZ2 domain of PTPL1 physically interacts with the NR2B subunit of NMDA-R.

[77] *NR2A interaction.* The interaction between the C-terminus of NR2A and the PDZ2 domain of PTPL1 was demonstrated in an experiment where cDNA encoding the C-terminal 600 amino acids of NR2A was inserted into the GAL4 BD plasmid (pAS2-1). This plasmid, along with the GAL4 AD plasmid (pACT2) which contains the PDZ2 domain of PTPL1, was transformed to Y187 yeast cells. Growth on selective medium was observed. This indicates that NR2A, the second most tyrosine-phosphorylated NMDA-R subunit in the brain, interacts with PTPL1.

"Pull-down" experiments

[78] "Pull-down" experiments demonstrating PTPL1/NMDA-R interaction are performed as follows. The portions of NR2A and NR2B containing the C-terminal 145 amino acids were expressed as fusion proteins with glutathione-S-transferase (GST) in *E. coli*. Bacterial cells from 25 ml LB medium harboring expressed proteins are lysed by sonication (10s) on ice, and bacterial debris pelleted by centrifuging the sonicate for 20 min at 15,000g. Expressed proteins are purified by adding the supernatant to 100 µl of 50% Glutathione-Sepharose-4B (Pharmacia) bead slurry in phosphate buffered saline (PBS), incubated by shaking for 30 min at 4°C. Non-specifically bound proteins are removed by three washes of beads with ice-cold PBS. The purified GST-NR2A and GST-NR2B proteins attached to the beads are mixed with the PTPL1 protein tagged with the c-myc epitope and heterologously expressed in 293/COS cells, and washed to remove non-specifically bound proteins. The binding of PTPL1 to the C-termini of NR2A or NR2B is determined by Western blotting using anti-c-myc antibodies (Clontech).

[79] For the negative control, the GST-NR2B fusion in which the valine residue in the very C-terminus is mutated to alanine is used. Furthermore, synthetic inhibitory peptides (KLSSIESDV) corresponding to the C-terminal nine amino acids of NR2A or NR2B are used for competition at a concentration of 0.5 mM to demonstrate the specificity of the interaction. For positive control, heterologously expressed post synaptic density 95 (PSD95, see, Niethammer et al., J. Neurosci. 16: 2157-63, 1996) is used in the similar set of experiments.

[80] In the reverse experiment, the GST fusion with the second PDZ domain of PTPL1 is expressed in *E. coli*, purified and used to bind both the heterologously expressed NR2A or NR2B as well as to capture NR2A or NR2B subunits from the rat brain lysate. The specific binding of NR2A or NR2B to GST-PTPL1 is detected by Western blotting using specific anti-NR2A or NR2B antibodies (Chemicon).

[81] For positive control, synthetic inhibitory peptides corresponding to the C-terminal nine amino acids of NR2A or NR2B (KLSSIESDV) are used for competition at a concentration of 0.5 mM to demonstrate the specificity of the interaction.

Co-immunoprecipitation

[82] Co-immunoprecipitation experiments demonstrating the NMDA-R/PTPL1 binding are performed as follows. The combinations of eukaryotic CMV promoter driven expression vectors that contain cDNAs encoding the following proteins are co-expressed in 293 cells in different combinations.

Full length clones:

1. NR1,
2. NR2A,
3. NR2B
4. PTPL1,
5. PTPL1-CS (inactive PTPase)

Deletion mutants:

1. NR2A C-stop (truncated NR2A subunit, does not contain c-terminus)
2. NR2B C-stop (truncated NR2B subunit, does not contain c-terminus)
3. c-myc PTPL1 wt-short (PDZ2-stop)
4. c-myc PTPL1 CS-short (PDZ2-stop)

[83] For all experiments, 7-10 micrograms of total plasmid DNA per semi-confluent dish of cells can be transfected by, e.g., calcium phosphate precipitation (Wigler M, et al., Cell 16:777-785, 1979). Cells can be harvested 48 hours post-transfection, the medium removed upon centrifugation and the cells resuspended in Lysis Buffer (150mM NaCl, 50mM Tris pH 7.6, 1% Triton). 200 μg lysate (1μg/μl) is incubated with 1-3 μg of primary antibody, overnight at 4°C, shaking.

[84] After co-incubation of antibodies and heterologously expressed proteins, 20 μl of Protein A/G Plus-Agarose (Santa Cruz) slurry is added, and the incubation is continued for another hour. To determine co-immunoprecipitated proteins, material bound to Protein AG-Plus Agarose is separated by pelleting the beads with the immunocomplex attached by centrifugation, washed with PBS and resolved by 4-12% SDS-PAGE. Proteins resolved on the gel are transferred to membrane to verify the presence of co-immunoprecipitated proteins by Western blots using specific antibodies as outlined above.

[85] The data show that HA-tagged full length PTPL1 co-precipitates with both NR2A and NR2B subunits. It does not interact with NR2A C-stop and NR2B C-stop, which do not contain the c-terminus with the interaction domain. Truncated PTPL1 clones containing PDZ and PTP domains (c-myc PTPL1 wt-short, and CS short) also co-precipitate with both NR2A and NR2B subunits.

Example 2

Characterization of PTPL1 and NMDA-R

Expression

[86] Using an antisense oligonucleotide (5'-CCATCACCCGCACCACGAAG CCCTTCAGCTGCTGCATTCTCA 3'), *in situ* hybridization studies were carried out to examine PTPL1 expression in rat brain. The results indicate that PTPL1 is expressed in all major neuronal populations in the adult rat brain. Thus, there is a very high degree of overlap between the cellular localization of PTPL1 and NMDA-R in the brain. In addition, *in situ* hybridization was performed using a rat PTPL1 cDNA riboprobe.

[87] *Animal Preparation and experimental Groups.* The procedures for transient MCAO were performed as described previously (Zhao *et al.* (1997) J Cereb Blood Flow Metab. **17**(12):1281-90) and are summarized briefly below. Male Wistar rats (Möllegaards Breeding Center, Copenhagen), weighing 310-350 g, were fasted overnight but had free access to water. Anesthesia was induced by inhalation of 3% halothane in N_2O:O_2 (70%:30%), whereafter the animals were intubated. They were then ventilated on 1.0-1.5% halothane in N_2O:O_2 during operation. The tail artery was cannulated for blood sampling and blood pressure monitoring. Blood pressure, PaO_2, $PaCO_2$, pH, and blood glucose were measured, and 0.1 ml of heparin (300 units×ml^{-1}) was given through the tail artery just before induction of ischemia. A surgical mid-line incision was made to expose the right common, internal, and external carotid arteries. The external carotid artery was ligated. The common carotid artery was closed by a ligature, and the internal carotid artery was temporarily closed by a microvascular clip. A small incision was made in the common carotid artery, and a nylon filament, which had a distal cylinder of silicon rubber (diameter 0.28 mm), was inserted into the internal carotid artery through the common carotid artery. The filament was further advanced 19 mm to occlude the origin of the middle cerebral artery (MCA). When the middle cerebral artery occlusion (MCAO) had been performed, animals were extubated and allowed to wake up and resume spontaneous breathing. In the group aimed for recirculation, the animals were reanesthetized with halothane after 2hrs of MCAO, and the filament was withdrawn. During the operation, an electrical temperature probe was inserted 7 cm into the rectum to monitor core temperature, which was regularly maintained at 37°C. After the operation, the animals were cooled by an air cooling system to avoid the hypothermia which would otherwise occur and to keep core temperature close to normal levels during and following MCAO. All animals were tested for neurological status according to the neurological examination grading system described by Bederson *et al.* (1986) Stroke **17**(3):472-6.

[88] Animals sacrificed after 2 h. of MCAO; or 3 min of ischemia for IPC and the time points as noted in Figures 1, 2 and 3. The brain were taken out and frozen in imbedding media at -50°C and stored at -80°C before sectioning.

[89] PTPL1 was examined by *in situ* hybridization. Tissue sections (15µm) were cut on a Microm cryostat and thaw-mounted on positively charged slides. After fixation with 4% paraformaldehyde (4°C, 5 minutes), sections were processed as followed: 1) washed 2 minutes in 0.1 mol/L phosphate buffer saline (PBS pH 7.2. 2) 0.1 M TEA 1 minute. 3) 0.25% acetic anhydride\TEA for 10 minutes. 4) Rinse 2 times in SSC. 5) Dehydrated in 70% (two minutes), 95% (two minutes) and 100% (two minutes) ethanol. 6) 5 minutes in chloroform and 2 minutes in 95% ethanol and finally air-dried for 10 minutes. A solution containing labeled probes was then contacted with the cells and the probes allowed to hybridize. Excess probe was digested, washed away and the amount of hybridized probe measured.

[90] The tissue from 2 h MCAO and 0, 1.5, 3, 6, 12, 24, and 48 hours recovery, and global ischemic preconditioning (IPC) (a model for tolerance to ischemic, see Shamloo and Wieloch (1999) J Cereb Blood Flow Metab 19(2):173-83) were generated and sectioned (3 min of ischemia (IPC) and 4h, 12h, 18h, 24h, and 48h). Also sectioned were 10 min of ischemia with or without IPC (2 days before the 10 min) and 12h, 18h and 48 h of recovery (after the 10 min). The tissue sections were processed and stored at AGY tissue bank.

[91] A PCR fragment was generated with SP6 and T7 promoter sequences for *in vitro* transcription (see Logel *et al.* (1992) Biotechniques 13(4):604-10. The amplified product was then used as a templicate for transcription to generate labeled mRNA, both sense and anti-sense. These probes were then used to hybridize to the tissue sections. Both sense and anti sense probes were generated and hybridized with MCAO or IPC tissues. Data were analyzed and information was stored.

[92] These results show upregulation of PTPL1 mRNA in global ischemia, as well as IPC, suggesting a protective role of PTPL1 in this disease models.

Immunocytochemistry

[93] In primary neuronal culture derived from the rat cerebral cortex and hippocampus, the studies of co-localization were conducted with the recombinantly expressed PTPL1. In such an experiment, a plasmid carrying cDNA construct (5 micrograms of DNA) encoding GFP-PTPL1 fusion protein, or an HA-tagged full-length PTPL1 was transfected to primary neurons using lipofection. The clustering of the GFP-PTPL1 fusion was observed in dendritic processes, which serve as input receivers from other cells and where NMDA-R are localized.

The co-localization of GFP-PTPL1 and NMDA-R can be demonstrated by immunocytochemistry using anti-NMDA-R antibodies.

[94] High resolution immunohistochemistry studies on brain slices (50-200 micrometers in thickness) are carried out to demonstrate the subcellular co-localization as described in *Antibodies*, Harlow & Lane, Eds., 1999. Using NR1- and PTPL1-specific antibodies to label endogenous NMDA-R and PTPL1 in neurons, the co-localization is detected by using antibodies derived from different species (such as rabbit or mouse; rabbit or goat etc.). The secondary antibodies which carry different reporters (e.g., different fluorescent tags) and specifically recognize antibodies from a particular species are used to differentiate between NMDA-R and PTPL1.

[95] *Antibody generation*. Two polyclonal antibodies against PTPL1 using oligopeptides (L1A (190) CSEQKPDRSQAIRDRLRGKGL and L1B (2362) CLEDIQTREVRHISHLNF) have been generated. Oligopeptide sequences were picked based on antigenicity prediction and an absence of potential glycosylation sites.

Modulation of NMDA-R signaling by PTPL1

[96] The following experiments are conducted to determine the role of PTPL1 in the modulation of NMDA-R signaling. Primary hippocampal neurons are transfected with or without PTPL1 and GFP as a marker using 5 micrograms of total plasmid DNA per well. The neurons co-expressing all components respond with the NMDA-R selective current when exposed to L-glutamate or NMDA. In order to measure NMDA currents, the cells are clamped with the patch pipette and characteristic NMDA-R currents recorded at different membrane potentials (Köhr & Seeburg, J. Physiol (London) 492: 445-452, 1996). Purified Src or Fyn is then allowed to diffuse to the cytosol of clamped cells through the patch pipette. Once again, the NMDA currents are recorded and the potentiation by the tyrosine kinases of NMDA-R currents is determined both in the presence and absence of transfected PTPL1.

[97] Alternatively, instead of applying purified Src or Fyn, a peptide, EPQ(pY)EEIPIA, that activates the members of Src family of tyrosine kinases is used to activate endogenous kinases in the cell and the NMDA-R currents are determined both in the presence and absence of transfected PTPL1.

[98] Patch clamp experiments with cells expressing NMDA-R and PTPL1 are carried out in the presence of 0.5 mM synthetic inhibitory peptides corresponding to the C-terminal nine amino acids of NR2A or NR2B (KLSSIESDV), as well as control peptides corresponding to the scrambled peptides with the same amino acid composition as the inhibitory peptide.

[99] Transfection of primary hippocampal neurons with HA-tagged full-length PTPL1 shows: a decrease in src mediated potentiation of synaptic NMDAR currents in presence of PTPL1, and a decrease in somatic NMDAR currents in presence of PTPL1. PTPL1 was expressed in primary neurons by transient transfection using Effectene reagent. Electrophysiological recordings were obtained from nucleated patches. This method allows recording of somatically localized NMDA receptors as opposed to synaptic receptor populations. In the presence of PTPL1 the NMDA receptor current was reduced by approximately 50%, normalized to AMPA receptors, *i.e.* glutamate receptors known to co-localize with NMDARs and not affected by PTPL1. These experiments confirm the results obtained on synaptic NMDA receptors. In addition, control experiments using a mutated (C-S) PTPL1 clone are used with an inactivated phosphatase domain.

De-Phosphorylation of NR2A or NR2B by PTPL1

[100] The following experiments are conducted to determine the role of PTPl1 in the modulation of NMDA-R signaling. Stable HEK293 cell lines (NR1+NR2A or NR1+NR2B) are transfected with constitutively active src kinase to obtain high phosphorylation of the NR2 subunits. Activity of src is monitored using phospho-specific src antibodies (PY418 and PY529). NR2 subunits are precipitated from the cell-lysate with an NR2A or NR2B specific antibody and src induced phosphorylation is detected with phosphospecific antibodies or a generic phosphotyrosine antibody using SDS-Page. In a similar experiment PTPL1 is co-transfected with src and should reduce either src phosphorylation or NR2A or NR2B phosphorylation. Both events lead to reduced NMDAR currents in the presence of PTPL1.

[101] Activation of intracellular src kinase in HEK293 cell can be obtained by stimulating serum starved HEK293 cells with growth factors (EGF, PDGF) at appropriate concentrations. Src activation is monitored by phosphospecific src antibodies (commercially available). Growth factor stimulation of the stable cell-lines in the presence or absence of PTPL1 will show increased or decreased (+PTPL1) NMDA-R phosphorylation, and activity.

Calcium Imaging

[102] The effect of modulating compound upon a NMDA-R is investigated by analysis of calcium flux through the channels upon activation or inactivation of the NMDA-R. A calcium imaging experiment is carried out as follows. Measurements are done in presence/absence of compounds in a stable cell line inducibly expressing NMDA-R subunits as described above by using a FLEX station/Flipper or Ca^{2+} Imaging (see Renard, S. *et al.* Eur. J.

Physicology 366:319-328 (1999)). The Molecular Devices FLEX station is a scanning fluorometer coupled with a fluid transfer system that allows the measurement of rapid, real time fluorescence changes in response to application of compounds. As the function of NMDA receptors depends critically upon their ability to act as calcium channels upon activation, the FLEX station in combination with calcium indicator dyes is used to measure NMDA receptor activity. This allows investigation of roles of interacting proteins in the modulation of both the magnitude and kinetics of NMDA receptor mediated calcium influx and screening for compounds that are able to modulate the functional properties of NMDA receptors. Stable cell lines inducibly expressing NMDA-R subunits are advantageous as they provide a homogenous population of cells, particularly useful for high throughput measurements in multi-well plate formats, which integrate the fluorescence properties of a population rather than individual cells.

Example 3

Screening for agents that modulate NMDA-R signaling

[103] *PTPL1 expression and purification.* A 1.2 Kb DNA fragment encoding PTPL1 residues G2067 through K2466 preceded by the tag MASHHHHHH was subcloned into the pET-17b vector (Novagen) between the NdeI and XhoI sites. The resulting plasmid was transformed into BL21(DE3) cells (Invitrogen), which were used for the expression of the PTPL1 catalytic domain (Ptase400). Cells were grown in LB medium at 37°C and induced at A_{600} = 0.6 with 0.1 mM IPTG for 3 hours before harvest.

[104] The cell paste was resuspended in 50 mM HEPES, pH 8.0 buffer containing 0.3 M NaCl, 1 mM PMSF, 1 mM β-mercaptoethanol, and 0.1% Triton X-100 and sonicated on ice. The cell lysate was centrifuged at 27,000 x g for 20 min, and the supernatant was loaded onto a Ni^{2+}-NTA (Qiagen) column equilibrated with 10 mM imidazole, 0.3 M NaCl, 50 mM HEPES, pH 8.0 buffer. The column was washed with the same buffer, and the protein was eluted with 200 mM imidazole, 0.3 M NaCl, 50 mM HEPES, pH 8.0 buffer.

[105] The eluate from the Ni^{2+}-NTA column was diluted 1:4 with 50 mM HEPES, pH 8.0 buffer and loaded onto a Q Sepharose Fast Flow (Pharmacia) column equilibrated with 50 mM MES, pH 6.2 buffer. The column was washed to baseline with 50 mM MES, pH 6.2 buffer and eluted with a salt gradient from 0 to 0.5 M NaCl over 30 column volumes. Fractions containing the Ptase400 were pooled and diafiltered into 100 mM NaCl, 100 mM Tris-HCl, pH 7.6 buffer.

[106] The protein obtained over the two chromatographies was at least 95% pure by Coomassie staining.

Assay Development

TR-FRET ASSAY

Material:

[107] Phosphatase Buffer: 50 mm HEPES, pH 8; 1 mM DDT; 2 mM EDTA; 0.01% Brij solution; 10 mM $MgCl_2$. Detection Buffer: 25mM Tris, pH 7.5 + 0.2% Trition 100; 0.5 µl Eu PY20 Ab; 1.5 µl Streptavidin-APC per 5 ml of Detection Buffer. *Buffers can be stored at 4° Celsius. Corning 384-well, assay plate 3617. Substrate: AGY 1336. Enzyme: PTPL1. Sodium Orthovanadate. DMSO (HPLC grade). Compound Plates: Compound plates are thawed overnight at room temp.

METHOD:

[108] The enzyme stock solution is made by adding 24.4 µl PTPL1 stock (at 1.9 mg/ml) to 100ml of phosphatase buffer. The substrate stock solution is made by adding 2 µL AGY-1336 (at 5 mM) to 100 ml of phosphatase buffer. The control inhibitor stock solution is made by adding 90 µl sodium orthovanadate (100µM) to 30 ml phosphatase buffer. The detection reagent stock solution is made by adding 15 µL Eu-anti-phosphotryosine antibody + 45 µL APC to 150 ml of detection buffer. This yields initial concentrations of: Enzyme: 10 nM; substrate: 100 nM; vanadate: 300 nM.

[109] The reagents for the control wells are dispensed by the Biomek 2000 (B2K) and Biomek FX robots. The B2K dispenses controls into six assay plates. 12.5 µl of enzyme, 2.5 µl of DMSO, and 10 µl of buffer is placed into column 1 and 2, rows A through H. A substrate volume of 12.5 µl, 2.5 µl of DMSO, and 10 µl of buffer is placed into columns 1 and 2, rows I through P. Column 23, row A through P will contain 5.0 µl of orthovanadate solution. Column 24 is left empty.

[110] For the enzyme activity assay, 2.5µl of compound, 12.5µl of enzyme, and 10µl of substrate (separated by air gaps) are added to columns 3 thru 24 by the Biomek FX in a single dispense. After the dispense, the tips are washed with DMSO and water for re-use between each quadrant. Once the assay plates are set up, they are incubated at 27° C for 45 minutes. Then 20µl of detection buffer is added to stop the reaction and to allow the Europium antibody (Eu-Ab) and streptavidin-APC to bind to the substrate.

[111] The plates are then placed in the plate reader, an Analyst HT. Excitation light at 360 nm is used to excite the Europium antibody with an emission at 620 nm. Fluorescence resonance energy transfer (FRET) from Eu-Ab to APC will only occur when they are in close proximity. Therefore, when an APC emission is observed at 665 nm the enzyme has been inhibited from removing the phosphate group from the substrate. The FRET assay is time-resolved (TR), where there is a delay between excitation light and collection of emission signals. This reduces the amount of stray light created by short-lived fluorescing molecules. The Analyst HT measures APC and Europium emission signals and calculates the ratio between the two intensities. Typical intensities for the Europium is ~2000 and APC is ~600.

[112] The specificity of inhibition is tested using a broad phosphatase panel to determine inhibition of phosphatases other than PTPL1. Once hits are identified as specific to PTPL1, the inhibitor is tested is secondary assays as described below, *e.g.* HEK293 cells expressing NR1/NR2A and NR1/NR2B subunits. Functional characterization of active compounds is performed in primary hippocampal neurons by electrophysiology. *In vivo* validation of PTPL1 inhibitors uses behavioural tests in mouse or rat animal models.

[113] *Design of profiling assays.* The development of secondary cell-based assays is used in the profiling of compounds. Key parameters of increased NMDAR activity include increased NR2 phosphorylation; increased NMDAR current; increased Ca^{2+} permeability. Transient expression of glutamate receptor subunits in HEK293 cells is used. The phosphorylation state of the NR2 subunits by endogenous kinases in HEK293 cells is determined, and tested for an effect on NMDA receptor activity.

[114] The profiling assays include transient expression of binary NR1/NR2B and NR1/NR2A receptor channels in the presence and absence of the agonist glutamate. Stable cell lines may also be used. Glutamate, by activating the NMDA receptor channels, also leads to an increased phosphorylation of the NR2 subunits and thus to increased current and Ca^{2+} permeability. Inhibition of endogenous phosphatases by orthovanadate inhibits endogenous phosphatases. Inhibition of endogenous kinases by genistein decreases NR2 phosphorylation and thus activity of PTPL1, by acting specifically on NR2 it decreases its phosphorylation and its activity. Identified compounds will specifically inhibit PTPL1 and lead to increased NR2 phosphorylation and Ca^{2+} influx upon NMDAR activation with glutamate. The functionality of NMDA receptors and their modulation is initially tested using calcium flux measurements. Different calcium indicator dyes are assessed.

[115] For profiling assays, primary hippocampal or cortical neurons are infected with either Sindbis or Lentivirus constructs expressing the wt PTPL1, PTPL1 (cs) and a GFP control.Organotypic cultures are also used. NMDA or L-Glutamate induced currents are recorded selectively in presence / absence of identified compounds. In order to measure NMDA currents, the cells are clamped with the patch pipette and characteristic NMDA-R currents recorded at different membrane potentials (Köhr & Seeburg, J. Physiol (London) 492: 445-452, 1996).

[116] Neuronal NMDA receptor function is measured using either electrophysiology or the FLEX station, i.e measuring Ca2+ influx. A calcium imaging experiment is carried out as follows. Measurements are done in presence/absence of compounds in a primary neuronal cell expressing NMDA-R subunits as described above by using a FLEX station/Flipper or Ca^{2+} Imaging (see Renard, S. *et al.* Eur. J. Physicology 366:319-328 (1999)). The FLEX station in combination with calcium indicator dyes is used to measure NMDA receptor activity. Similarly to the experiments in HEK293, it is expected to see a decrease in NMDAR current in neurons infected with the wt PTPL1 virus. Compounds would restore NMDAR function/activity by inhibiting PTPL1. The PTPL1 (cs) mutant serves as a control.